Pestic. Sci. 1997, **51**, 309–314

New Tools for Studying Epidemiology and Resistance of Grape Powdery Mildew to DMI Fungicides*

Christophe Délye,[1]† Frédéric Laigret[2] & Marie-France Corio-Costet[1]

[1] Unité de Recherches Intégrées sur la Vigne, Institut National de la Recherche Agronomique, Domaine de la Grande Ferrade, BP 81, 33883 Villenave d'Ornon cédex, France
[2] Laboratoire de Biologie Cellulaire et Moléculaire, Institut National de la Recherche Agronomique, Domaine de la Grande Ferrade, BP 81, 33883 Villenave d'Ornon cédex, France

(Received April 1997; revised version received 4 June 1997; accepted 10 July 1997)

Abstract: Using a Random Amplified Polymorphic DNA (RAPD) assay, we investigated the genetic polymorphism existing among 62 European isolates of the grape powdery mildew fungus (*Uncinula necator* [Schw.] Burr.). Isolates overwintering as mycelium in buds were genetically distinct from isolates overwintering as ascospores, suggesting the existence of two genetically isolated powdery mildew populations, and consequently of two independent sources of inoculum in the vineyard. Isolates resistant to fungicides inhibiting sterol 14α-demethylation (DMIs) were found in both populations, suggesting that resistance to DMIs may arise independently in the two powdery mildew populations. A PCR assay targeting the gene encoding *U. necator* 14α-demethylase has been developed which will permit an early, specific detection of *U. necator* infections, and may be useful for spraying programmes.

Pestic Sci., **51**, 309–314, 1997
No. of Figures: 2. No. of Tables: 2. No. of Refs: 18

Key words: PCR, detection, grape powdery mildew, demethylase, field resistance, inoculum

1 INTRODUCTION

Grape powdery mildew is a major disease of grape (*Vitis vinifera*, L.) in vineyards throughout the world. The causal agent is *Uncinula necator* (Schw.) Burr., an obligate biotrophic ascomycete fungus. In the absence of fungicide treatment, this pathogen may cause heavy damage. Although *U. necator* has been present in European vineyards for more than 150 years,[1] little is known about its epidemiology. Because of the lack of substantial epidemiological data, systematic fungicide treatments are employed for disease control. These have caused the build-up of resistance to sterol 14α-demethylation inhibitors (DMIs), the major class of fungicides used on grape in European and Californian vineyards in the late 1980s.[2,3] Nowadays, resistance of grape powdery mildew to DMIs is widespread throughout the vineyards where these compounds are used. Genetic control of resistance seems to be polygenic,[4] although resistance mechanisms remain unknown.

Two main sources of primary inoculum may co-exist in the vineyard. One consists of ascospores, the sexual spores of the fungus, which are released from budburst to bloom of grapevines by cleistothecia that have overwintered on the grape plant's corky bark.[5–7] The other is asexual spores (conidia) released from heavily sporulating infected grape shoots called 'flagshoots'.[8] These symptoms are caused by mycelium overwintering in dormant buds, which resumes activity after budbreak.[9,10] It is still not known whether the same genotypes of *U. necator* may overwinter as mycelium in buds

* Based on a presentation at the Conference 'Resistance '97—Integrated Approach to Combating Resistance' organised by the Institute of Arable Crops Research in collaboration with the SCI Pesticides Group and the British Crop Protection Council and held at Harpenden, Herts, UK on 14–16 April 1997.

† To whom correspondence should be addressed.

 Pestic. Sci. 0031-613X/97/$17.50. Printed in Great Britain

and as cleistothecia, or whether different biotypes of the fungus exist. This point is of importance for disease control strategies in vineyards where both cleistothecia and 'flagshoots' are present.

To obtain molecular markers that may be (i) used for epidemiological studies of grape powdery mildew in the vineyard, such as markers specific to a particular isolate or to a geographical origin, and/or (ii) correlated with resistance to DMIs, we conducted a RAPD (Random Amplified Polymorphic DNA)[11] study on 62 European isolates of grape powdery mildew. These included nine isolates obtained from typical 'flagshoots' symptoms, which were collected in vineyards where DMI resistance was either detected or, at least, suspected. The sensitivity of all isolates to triadimenol was assessed, and cross-resistance to various DMIs of eight isolates, including two isolates from 'flagshoots', was investigated.

Because early detection and treatment of primary infection of grape by powdery mildew in the vineyard may check, or significantly delay, an epidemic, we developed a PCR-based assay targeting the gene encoding *U. necator* eburicol 14α-demethylase, which allows the specific detection of this fungus on crude samples from the vineyard.

2 EXPERIMENTAL METHODS

2.1 *Uncinula necator* isolates

Sixty-two clonal isolates of *U. necator* from Europe were used in this study. Except for the nine 'flagshoot' isolates, which were all collected in April 1994 from two locations in France and one in Germany, isolates were collected between 1988 and 1994 from May to September in vineyards where grape powdery mildew was not fully controlled by fungicide treatments. Isolates were collected in France (36 isolates, including five isolates from 'flagshoots'), Portugal (11 isolates), Switzerland (seven isolates) and Germany (eight isolates, including four isolates from 'flagshoots'). The fungus was maintained on the upper surface of young leaves from the grape cultivar 'Cinsaut' previously decontaminated by immersion (10 min) in calcium hypochlorite (50 g litre^{-1}), rinsed, dried between two autoclaved paper sheets and placed in Petri dishes (9 cm diameter) containing water agar (20 g litre^{-1}; 18 ml). Clonal isolates were obtained by picking a single powdery mildew conidium from mildewed samples within a laminar flow hood using an eyelash fastened to a holder. After incubation (14 days, 22°C, 1000 lux illumination 16 h day^{-1}), *U. necator* isolates were inoculated under sterile conditions on the surface of four decontaminated grape leaves placed inside a Plexiglas inoculation tower (25 × 25 cm × 60 cm high) by blowing conidia from sporulating leaves using an aquarium pump connected to a flexible plastic tube (3 mm diameter) terminating in a Pasteur pipette. Inoculated leaves were incubated as above.

2.2 Fungicide testing

Fungicide testing was as described elsewhere.[4] The fungicides used were triadimenol (Baytan 5L, 50 g litre^{-1} EW, Bayer S. A., Puteaux, France), triadimefon (Bayleton, 50 g kg^{-1} WP, Bayer S. A.), penconazole (Topaze, 100 g litre^{-1} EC, Ciba, Rueil-Malmaison, France), tebuconazole (Corail, 250 g litre^{-1} EW, Bayer S. A.), fenbuconazole (Indar, 50 g litre^{-1} EC, Rohm & Haas France S. A., Paris, France), cyproconazole (Atemi 10 Pépite, 100 g kg^{-1} WG, Sandoz Agro, St-Germain-en-Laye, France), pyrifenox (Dorado, 200 g litre^{-1} EC, La Quinoléine et ses dérivés S. A., Rueil-Malmaison, France) and fenarimol (Rubigan 4, 40 g litre^{-1} EC, Dow Elanco S. A., Sophia Antipolis, France). Fungicides were kindly donated by the manufacturers and used at 0·0, 0·1, 0·3, 0·5, 0·8, 1·0, 2·0, 5·0 and 10·0 mg AI litre^{-1} in each case. Inhibition of fungal growth was determined by assessing the percentage of leaf disc surface covered with sporulating powdery mildew for each fungicide concentration using a binocular microscope. Tests were duplicated with a range of concentrations adapted to the response of each isolate in order to obtain a dose-response curve (growth inhibition against fungicide concentration). Fungicide concentrations causing 50% (IC_{50}) and 100% (MIC, minimal inhibitory concentration) inhibition of growth of isolates, expressed as μM, were determined graphically from these curves, to allow comparison between compounds. An isolate was considered resistant if its MIC was $>4{\cdot}5$ μM (1·5 mg litre^{-1}) for fenarimol, 3·4 μM (1·0 mg litre^{-1}) for triadimenol, triadimefon and penconazole, 3·2 μM (1·0 mg litre^{-1}) for tebuconazole, 3·0 μM (1·0 mg litre^{-1}) for fenbuconazole or 1·0 μM (0·3 mg litre^{-1}) for cyproconazole and pyrifenox. Resistance factors (RF) were calculated for resistant isolates as: RF = IC_{50} of the isolate/mean IC_{50} of all isolates sensitive to the fungicide studied, because DMI sensitivity also varied among sensitive isolates.

Sensitivity of the 62 isolates of *U. necator* to triadimenol was assessed. Cross-resistance patterns of eight isolates, including two 'flagshoot' isolates, were also investigated for at least two other DMIs.

2.3 RAPD assays

Collection of fungal material, DNA extraction, and amplification were performed as described elsewhere.[12] Forty-six RAPD primers which revealed polymorphism between *U. necator* isolates were used in this study (the list of these primers is available from the authors upon request). At least two independent amplifications, using

two different DNA samples, were performed for all primer and isolate combinations.

2.4 PCR assays

Primers C14 and C14R, which correspond to the flanking sequences of the 1683 bp gene encoding eburicol 14α-demethylase of *U. necator* (Genbank accession No. U72657),[13] were used to develop a specific detection assay based on PCR. These primers were designed to generate a 1756-bp-long PCR fragment. Their sequence is available from the authors upon request. PCR reaction mixtures (20 μl) contained Tris-HCl (70 mM), $MgCl_2$ (2 mM), $(NH_4)_2SO_4$ (17 mM), β-mercaptoethanol (10 mM), polyoxyethylene-ether W1 (0·5 g litre^{-1}, Sigma Chemicals, St Louis, USA), bovine serum albumin (0·2 mg ml^{-1}), dATP, dCTP, dGTP and dTTP (200 μM each), approximately 10 ng of template DNA, Goldstar DNA polymerase (0·5 unit, Eurogentec S. A., Seraing, Belgium) and 0·2 μM each of primers C14 and C14R. Reaction mixtures were submitted to 37 cycles of PCR amplification, each consisting of 30 s denaturation at 94°C, 1 min annealing at 65°C and 1·5 min extension at 72°C, in a DNA thermocycler (Perkin-Elmer Cetus, Norwalk, USA). To ensure that amplification was specific for *U. necator*, PCR amplifications were also performed with DNA extracted from grape and from various saprophytic fungi which grew on PDA plates in which non-decontaminated grape leaves, collected in the vineyard, were placed. In order to estimate the sensitivity of this assay, freeze-dried fungal material from grape powdery mildew and from fungi which grew on PDA plates were mixed in the proportions 1 + 1, 1 + 10, 1 + 100, 1 + 300, 1 + 500, 1 + 700 and 1 + 1000 (by weight) before DNA extraction and PCR amplification. Amplifications were performed three times with three different DNA extractions.

3 RESULTS AND DISCUSSION

3.1 Fungicide testing

3.1.1 Sensitivity to triadimenol

Of the 62 isolates studied, which were sampled in vineyards where resistance was reported or suspected, 29 were sensitive and 33 resistant to triadimenol. MICs of sensitive isolates ranged from 1·3 to 2·9 μM triadimenol, whereas those of resistant isolates ranged from 8·5 μM to >50·0 μM triadimenol. IC_{50} values of the sensitive isolates ranged from 0·50 to 0·85 μM triadimenol, the mean IC_{50} value for the 29 sensitive isolates being 0·75 μM triadimenol. RF values of resistant isolates ranged from 2·7 to 29·5. The data in Table 1 show that the most resistant isolates of *U. necator* displayed RF values between 2 and 10 (26 isolates out of 33), but seven isolates exhibited RF values >10, and two displayed RF values >25 (28·6 and 29·5, respectively). This range of RF values confirmed that DMI resistance in *U. necator* is not simple. Eight isolates, displaying contrasting IC_{50} values, were chosen for further fungicide testing.

TABLE 1
Resistance Factors (RF)[a] of 62 *Uncinula necator* Isolates to Triadimenol

Triadimenol sensitivity	*Number of isolates*
Sensitive[b]	29
$2 < RF \leqslant 5$	16
$5 < RF \leqslant 10$	10
$10 < RF \leqslant 15$	3
$15 < RF \leqslant 20$	2
$20 < RF \leqslant 25$	0
$25 < RF \leqslant 30$	2

[a] Resistance factor ($IC_{50\,res}/IC_{50\,sens}$; $IC_{50\,sens} = 0{\cdot}75$ μM triadimenol).
[b] MIC < 3·4 μM (1 mg litre^{-1}) triadimenol.

3.1.2 Cross-resistance patterns of eight isolates to various DMIs

Cross-resistance patterns were established for six isolates collected between May and September, three in France (isolates FPE11, FIO12 and FMA21) and one each in Portugal (isolate PTV12), Switzerland (isolate SNO11) and Germany (isolate GUN13), and for 'flag-shoot' isolates collected in France (isolate FIO21) and in Germany (isolate GNE11). The results of fungicide tests are presented in Table 2. A variety of IC_{50} values were obtained, depending both on the isolate and on the fungicide tested. No consistent cross-resistance to the DMIs tested was observed, as was previously shown for other DMI-resistant fungi.[14,15] However, isolates highly resistant to triadimenol (isolates SNO11 and FMA21) had a tendency to be at least weakly resistant to other compounds. Isolate PTV12, which was weakly triadimenol-resistant, was also resistant or weakly resistant to most of the other compounds tested.

3.2 RAPD amplifications

The 46 RAPD primers tested on the 62 isolates yielded 382 RAPD fragments, 79 (20·7%) of which were polymorphic. All RAPD patterns obtained were completely reproducible. The sizes of the amplified DNA fragments ranged from 0·2 to 3 kilobase pairs. A total of eight 'rare' RAPD fragments (fragments found exclusively in one isolate or one group of isolates displaying identical RAPD patterns) were found among the 62 isolates. No fragment was specifically present or absent in the RAPD patterns of all triadimenol-resistant isolates. A Fisher exact test was consequently used to determine

TABLE 2
Sensitivity of Eight Isolates of *Uncinula necator* to Various DMIs

Compound	IC_{50}[a] *of isolate* (μM) *FPE11*	*FIO12*	*FMA21*	*PTV12*	*SNO11*	*GUN13*	*FIO21*[b]	*GNE11*[b]
Triadimenol $IC_{50\ sens}$[c] = 0·75 μM	0·35 (S[d])	0·70 (S[d])	11·25 (15·0)	2·05 (2·7)	21·50 (28·6)	0·80 (S[d])	5·90 (7·9)	0·85 (S[d])
Triadimefon $IC_{50\ sens}$[c] = 0·70 μM	0·50 (S[d])	1·45 (2·1)	4·95 (7·1)	7·00 (10·0)	18·00 (25·7)	2·05 (2·9)	8·00 (11·4)	0·40 (S[d])
Penconazole $IC_{50\ sens}$[c] = 0·90 μM	0·85 (S[d])	nd[e]	1·10 (1·2)	nd	2·00 (2·2)	nd	nd	nd
Tebuconazole $IC_{50\ sens}$[c] = 0·50 μM	nd	0·30 (S[d])	nd	1·00 (2·0)	nd	nd	1·65 (3·3)	0·65 (S[d])
Fenbuconazole $IC_{50\ sens}$[c] = 0·35 μM	nd	0·30 (S[d])	nd	0·30 (S[d])	nd	nd	0·40 (S[d])	nd
Cyproconazole $IC_{50\ sens}$[c] = 0·10 μM	0·05 (S[d])	0·10 (S[d])	0·50 (5·0)	1·00 (10·0)	0·35 (3·5)	0·10 (S[d])	0·85 (8·5)	0·05 (S[d])
Pyrifenox $IC_{50\ sens}$[c] = 0·15 μM	nd	0·15 (S[d])	nd	0·75 (5·0)	nd	nd	0·75 (5·0)	0·10 (S[d])
Fenarimol $IC_{50\ sens}$[c] = 0·95 μM	0·80 (S[d])	0·75 (S[d])	0·90 (S[d])	2·65 (2·8)	nd	nd	1·00 (S[d])	0·75 (S[d])

[a] Concentration of compound inhibiting 50% of fungal growth. Values in parentheses are resistance factors ($IC_{50\ res}/IC_{50\ sens}$).
[b] 'Flagshoot' isolates.
[c] Mean IC_{50} value computed for all isolates sensitive to the compound.
[d] Sensitive isolate.
[e] nd = Not determined.

whether polymorphic fragments showing a significant frequency difference between the sensitive isolates group and the resistant isolates group could be revealed. Such fragments were not obtained.

The isolates could be divided into two very distinct groups according to their RAPD patterns (Fig. 1). The first group contained the nine isolates from 'flagshoots', while the second group was made up of the remaining 53 isolates collected between May and September. No isolate displaying RAPD patterns similar to those of 'flagshoot' isolates was found among these 53 isolates, although some of the samplings were performed in vine-

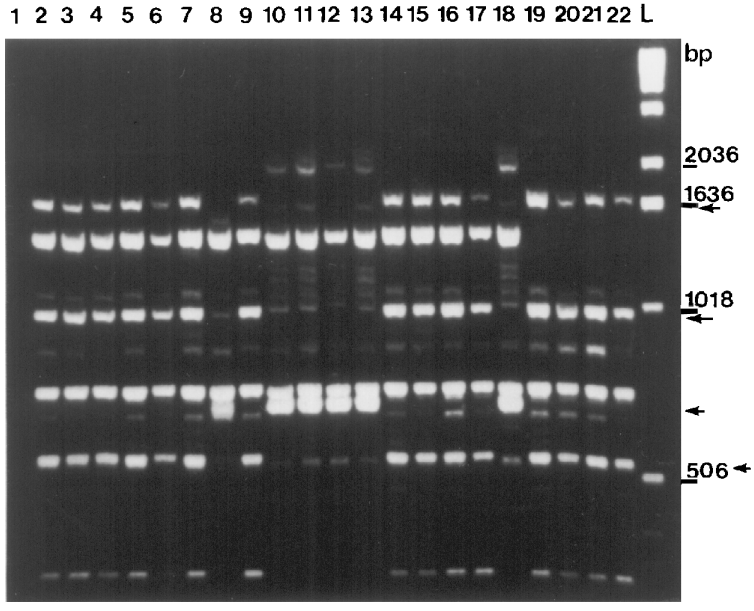

Fig. 1. RAPD profiles of 21 isolates of *Uncinula necator* obtained with primer OPJ-1 (5′-CCCGGCATAA). RAPD patterns are shown for DNA extracted from five 'flagshoot' isolates from France (lanes 8 and 10–13) and one from Germany (lane 18), and from 15 isolates collected in France (lanes 2–7, 9 and 14), Portugal (lanes 15–17), Germany (lanes 19–20) and Switzerland (lanes 21–22) between May and September. Fragments specifically present or absent in the RAPD patterns of 'flagshoot' isolates are arrowed. Lane 1: H_2O negative control (no DNA). Lane L: molecular weight marker (1 kb DNA ladder, Gibco-BRL, Gaithersburg, USA).

yards where both 'flagshoots' and cleistothecia were present. Two consecutive samplings were performed on the same grape plants, which displayed both 'flagshoot' symptoms and cleistothecia, during the same growing season in two vineyards in France and in one vineyard in Germany. The first samplings were performed in April, and the second during summer (July–August). April samplings yielded the nine 'flagshoot' isolates with particular RAPD patterns, whereas summer samplings yielded a total of 17 isolates, all displaying RAPD patterns similar to those of the isolates collected between May and September throughout Europe (Fig. 1).

These data suggest that, in the vineyards investigated where both 'flagshoots' and cleistothecia were present, a shift may have occurred between April and July–August from a population mainly consisting of 'flagshoot' isolates to a population mainly consisting of 'cleistothecia' isolates. The absence of 'flagshoot' isolates in the samples collected in July and August suggests that these isolates represented only a small proportion of the powdery mildew population at that time, or were no longer actively growing in the vineyards. This is in agreement with a previous study showing that bud infection by *U. necator* occurs early in the season.[10] It is possible that 'flagshoot' isolates appear early in the season, infect buds within a few weeks, and resume dormancy until next budbreak.

The RAPD patterns of 'flagshoot' isolates displayed 291 fragments, 5 (6·8%) of which were polymorphic. They shared 31 fragments that were not present in the RAPD patterns of any other isolate. The 53 other isolates displayed a total of 337 RAPD fragments, 75 (22·2%) of which were polymorphic. They shared 38 fragments that were absent in the RAPD patterns of all 'flagshoots' isolates.

The high number of RAPD fragments discriminating 'flagshoot' isolates from the other isolates suggests that no genetic recombination, and consequently no sexual reproduction, occurs between 'flagshoot' and 'cleistothecia' isolates. These two sources of inoculum are therefore probably evolving independently, which implies specific disease-management strategies. DMI-resistant isolates have been found among both 'flagshoot' isolates and 'cleistothecia' isolates, suggesting that resistance mechanisms are likely to arise independently in both populations of *U. necator*.

The great number of RAPD fragments discriminating 'flagshoot' conidial isolates from 'cleistothecia' isolates will enable the development of molecular tools such as specific PCR-SCAR (Sequence Characterised Amplified Regions)[16] primers that will be used to monitor field populations of *U. necator* to corroborate the hypothesis that a succession of powdery mildew populations occurs in the vineyards where both 'flagshoots' and cleistothecia are present. If 'flagshoot' isolates would not actively grow during summer in the vineyards, a single treatment performed early in the season should be sufficient to stop the epidemic caused by this source of inoculum, without selecting 'flagshoot' isolates for resistance. However, additional data are needed to determine whether or not isolates derived from sexual reproduction are capable of overwintering as mycelium in dormant buds.

3.3 PCR detection of *Uncinula necator*

PCR amplifications performed using specific primers derived from the sequence of the 14α-demethylase gene of *U. necator* on DNA extracted from isolates of this fungus yielded a single DNA fragment of the expected size (Fig. 2). No amplification was obtained with DNA extracted from grapes (not shown) or from fungi which grew on PDA plates (Fig. 2). Amplification of the 1756 base pairs PCR fragment was repeatedly obtained for DNA extracted from mixtures of freeze-dried fungal material from grape powdery mildew and saprophytic fungi ranging from 1 + 1 to 1 + 500 in respective proportions (Fig. 2). Amplification of this fragment in the 1 + 700 proportion mixtures was weak, and not clearly obtained in all assays. The lower limit for the detection of *U. necator* was thus found to be one part of powdery mildew fungal material in 500 of saprophytic fungi.

Primers C14 and C14R proved to amplify specifically DNA from *U. necator*, and to permit the detection of very small amounts of powdery mildew mixed with various saprophytic fungi that are present on grape leaves in the vineyard. Early detection of *U. necator* in the vineyard using PCR is thus achievable. Because the technique used to obtain DNA from fungal material present on the surface of grape leaves is fast,[12] DNA

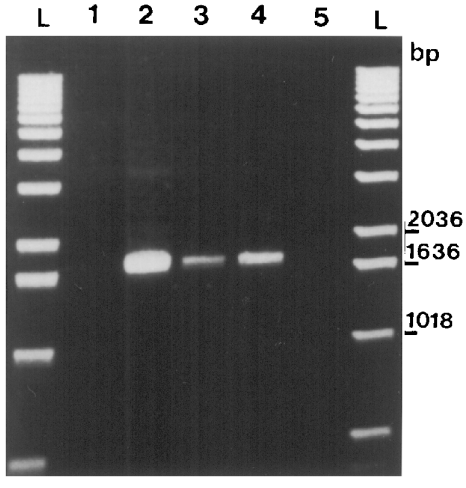

Fig. 2. PCR amplifications performed with primers C14 and C14R on DNA extracted from a mixture of various saprophytic fungi which grew on PDA plates from grape leaves collected in the field (lane 1), from grape powdery mildew (lane 2), from mixtures of fungal material from grape powdery mildew and from saprophytic fungi 1 + 500 and 1 + 100 (w/w) respective proportions (lanes 3 and 4). Lane 5: H_2O negative control (no DNA). Lane L: molecular weight marker (1 kb DNA ladder, Gibco-BRL, Gaithersburg, USA).

extraction, PCR amplification and assessment of the presence of PCR products can be achieved in the same day for a high number of samples.

4 CONCLUSIONS

No RAPD fragment specific to all DMI-resistant isolates or correlated with resistance could be obtained in our study. However, because genetic control of resistance to DMIs seems to be polygenic, cloning and sequencing the four 'rare' RAPD fragments that are specific to one resistant isolate could provide information concerning resistance mechanism(s) in these isolates. RAPD fragments specific to a single genotype of *U. necator* ('rare' fragments) are also of interest to develop molecular tools to assess gene flow between *U. necator* populations. Using a range of different SCAR-PCR primer pairs should make it possible to monitor the dispersal, survival and the consequences of possible sexual reproduction of a particular isolate of *U. necator* released in a vineyard with indigenous powdery mildew populations. Assessing the importance of sexual reproduction of *U. necator* in the field is of importance to understand the evolution of the polygenic resistance of this fungus to DMIs.

The numerous RAPD fragments discriminating 'flagshoot' and 'cleistothecia' isolates are of interest to develop a range of SCAR-PCR primers in order to monitor *U. necator* populations in the field. Such specific PCR primers may be used to perform amplifications directly on DNA extracted from crude field samples without the need to obtain pure isolates. This will allow the study of a large number of samples to obtain data concerning the biology and the epidemiology of *U. necator*.

The successful amplification of a PCR fragment encompassing the gene encoding *U. necator* eburicol 14α-demethylase will enable cloning and sequencing of this gene from powdery mildew isolates sensitive or resistant to DMIs. This approach may allow the detection of mutation(s) in this gene correlated with resistance to DMIs. To date, the only mutation found in the gene encoding sterol 14α-demethylase of a phytopathogenic fungus that may cause resistance to DMIs has been found in mutant isolates of *Penicillium italicum* Wehmer resistant to DMIs.[17] The different levels of resistance to various DMIs found among isolates of *U. necator* could be due to the presence of different mutations in this gene, as shown elsewhere for resistance to benzimidazole fungicides,[18] and/or to other mechanisms that remain to be elucidated. Understanding the molecular basis of such mechanisms will be of great help in developing molecular markers for monitoring field resistance to existing fungicides, as well as to determine strategies of use for new molecules.

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